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A Modular Analyzer System for Double Antibody Radioimmunoassays[1])

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Summary: The increasing number of determinations performed by radioimmunoassays necessitates rationalization of the procedures. An analyzer system has been developed in order to fully mechanize double antibody radioimmunoassays, which is essentially composed out of four independently working modules. The samples, in microliter vials, are carried in sample chains of up to 650 links. The first pipetting step is performed by syringes with displacement pistons. Additional reagents are rapidly added by an electronically controlled Hamilton repeating dispenser, which makes shaking procedures for mixing unnecessary. The bound/free separation is achieved discontinuously by use of Nuclepore-filters, which are carried in 3 inches distance (76 mm) by a 35 mm dark leader film. After covering the radioactive filter positions with an adhesive plastic foil from both sides, the film spool is directly inserted into a specially constructed gamma-counter. The results of the evaluation of the efficiency and of the precision of each module are presented in this paper.

Ein modulares Analysensystem für Doppelantikörper-Radioimmunoassays

Zusammenfassung: Die wachsende Anzahl radioimmunologischer Bestimmungen erfordert die Rationalisierung der Arbeitsgänge. Zur Vollmechanisierung von Doppel-Antikörper-Radioimmunoassays wurde ein Analysensystem entwickelt, das aus vier unabhängig voneinander arbeitenden Modulen besteht. Als Probenträger werden Mikrolitergefäße in Reaktionsketten bis zu 650 Gliedern verwendet. Der erste Pipettierschritt wird mit Verdrängungskolben-Dosiervorrichtungen vorgenommen, weitere Reagentien werden unter Verwendung einer elektronisch gesteuerten Hamilton-Repitierspritze in scharfem Strahl zugefügt, was Schüttelvorgänge erübrigt. Zur bound/free-Trennung wird diskontinuierlich über Nuclepore-Membranen filtriert, die in 3 Zoll Abstand (76 mm) von einem 35 mm Vorspannfilm getragen werden. Die radioaktiven Filterpositionen werden beidseitig zur Vermeidung von Kontamination mit Klebefolie abgedeckt. Die Film-Auffangspule kann direkt in einen eigens konstruierten Gamma-Zähler eingelegt werden. Die Ergebnisse der Präzisionsprüfung und die Leistung der einzelnen Module werden in der Arbeit aufgeführt.

Introduction

Approximately 6000 radioimmunological determinations are performed weekly in our laboratories, of which 80 % are double antibody assays. Therefore, we were primarily interested in the developement of an analyzer system, which would provide mechanization for the double antibody methods. The intention was to design a machine with sufficient flexibility, so that manually performed assays could be adapted without markedly altering the reaction-procedures, solutions and incubation times.

Among the different chemical and physico-chemical methods of the bound/free separation, the double antibody procedure is especially suitable for automation purposes. When the precipitating second antibody is not linked to carrier particles, as it is the case in certain solid phase assays, shaking procedures are unnecessary. Generally, shaking is difficult to realize in a continuously working system. In our system the discontinuous filtration replaces the centrifugation and its various manipulations as loading and emptying of the centrifuge and sucking off or decanting the supernatant (1).

The analyzer system includes four modules:

1. A diluter system, to pipet serum and a reagent, which is either the first antibody in non-equilibrium conditions or tracer in equilibrium conditions.
2. A pipetting unit for the addition of constant quantities of further reagents, e.g. tracer, first or second antibody.

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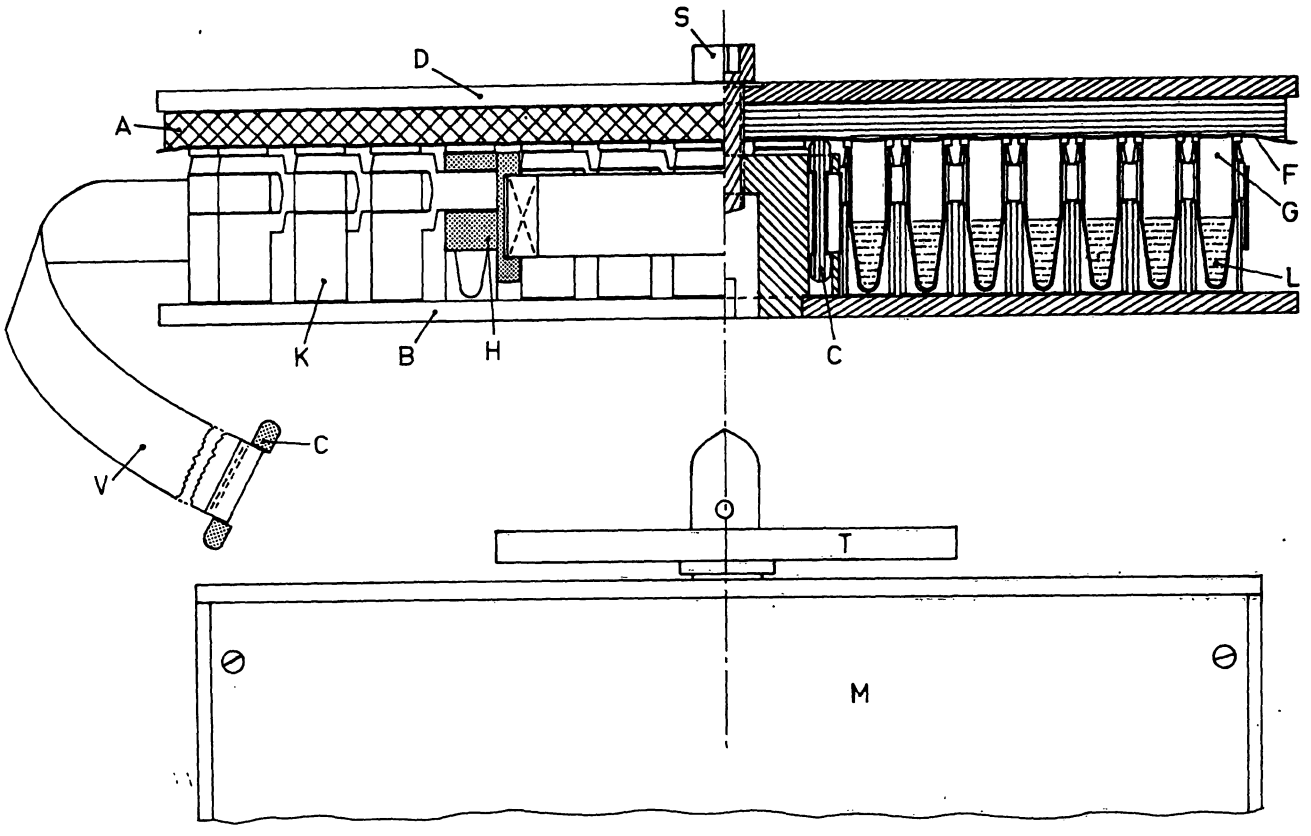

Fig. 1. Sample chain during incubation.

On the left of the axis the frontal view is depicted, on the right the sectional view, in the lower part the induction motor.
A = foam rubber, adhesive to the cover plate. B = base plate. C = clip holding the leader and the ending band. D = cover plate. F = plastic foil. G = open microliter vial. H = linkage between chain and leader. K = chain link. L = incubating sample. S = screw, pressing the cover plate on the vials. V = ending band. M = casing of the induction motor. T = support for the base plate B.

3. The filtration unit.
4. The specially constructed gamma-counter.

Preliminary results have been presented at the 21st Symposium der Deutschen Gesellschaft für Endokrinologie (2).

Materials and Methods

Description of the Modular Analyzer System

The first two modules of the system are not specific for separation-techniques and may be universally used. The sample carrier used for the first three modules is a commercially available chain, containing open microliter vials. These chains are coiled on discs with a diameter of 30 cm for 300 links, or 50 cm for 650 links. During the incubation (fig. 1) the chains are covered with one plastic foil and closed with one common screw cover. A 35 mm dark leader film is used as filter carrier. It contains Nuclepore-membranes of 20 mm diameter and 0.2 µm pore size at distances of three inches. One film has the length of 1000 feet (304.80 m) and carries 4000 filters. The chains and the film are the connecting links between all four modules of the system.

Diluter system

The diluter system[2]) uses the double chain principle. Four induction motors cause an even winding and unwinding of the chains (fig. 2). The primary chain contains the standards and the sera, succesively; the secondary chain holds empty vials. The sample is sucked into the needle by a syringe with displacement piston. Thereafter, the pipetting arm moves to the secondary chain and the sample is ejected into the vial, followed by the selected reagent through the same needle. The pipetting volume of both syringes is infinitely adjustable and may be set digitally. The number of replicates may be varied from 1 to 9. Three displays show the sample numbers of the primary and the secondary chain and the number of the replicate, which is actually pipetted. The dead volume of both syringes and of their tubings together is approximately 2 ml. The initial filling of the syringes is performed automatically by pressing a special button.

During incubation periods, the discs carrying the sample-chains are removed from the motors, covered as mentioned above and stored at room temperature or 4 °C, depending on the assay.

Pipetting Unit

The pipetting unit[2]) permits the addition of tracer, or first or second antibody (fig. 3). It employs the Hamilton[3]) repeating dispenser as dosage equipment. The volume may be varied between 50, 100 and 200 µl per piston stroke by using three different sizes of syringes. The ejection of the reagent is fast enough for perfect mixing in the vials. A highly precise toothed rack guarantees that the piston strokes are constant. The dead volume of the syringe and the tubing is 0.4 ml. The syringes of the diluter and the pipetting unit are fixed with bayonet fittings to the casings, which allows fast changing from one reagent to another.

[2]) RIA-E 6000, ISMATEC, CH-8031 Zurich, Switzerland.

[3]) Hamilton Micromesure B.V., The Hague, Holland.

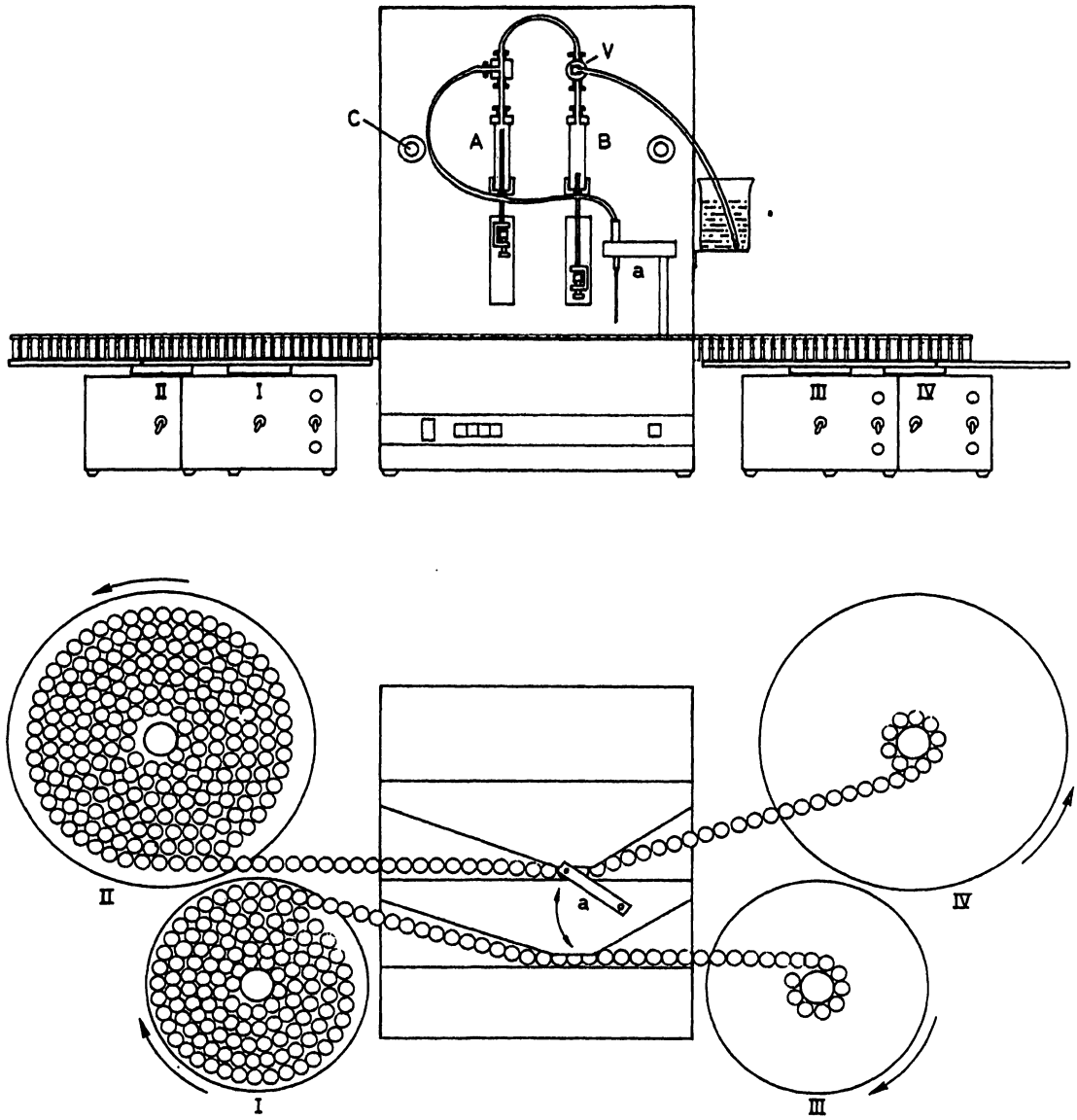

Fig. 2. Diluter system, frontal and plan view.

I–IV = induction motors with discs and sample chains. a = pipetting arm with needle. A = dosage syringe for the sample volume. B = dosage syringe for the buffer volume. C = infinitely adjusting for the pipetting volume. V = slide-valve.

The arrows show the direction of the traction of the induction motors. The transport of the chains is performed at the pipetting place by means of cogwheels (not shown in the figure).

Filtration Unit[2])

The incubation mixture is separated into antibody-bound and free fractions by filtration through Nuclepore[4]) filters with a pore size of 0.2 µm. Figure 4 shows schematically one filtration place. By means of the peristaltic pump P 2 the sample is pumped onto the filter membrane, through which the fluid phase is sucked by a vacuum pump. A concavely cut porous glass disc is used as support for the filter. The filter position is pressed onto the support. With the peristaltic pump P 1, the vials are washed twice with adjustable volumes of buffer containing 2 g/l albumin. The washing solution is likewise passed through the filter. The speed of the transport of the volumes passed and the duration of one filtration cycle, respectively, may be varied by altering the velocity and the time set to each of the two peristaltic pumps. At the end of the filtration cycle, the vacuum is switched off by an electric valve and the space below the filter is reventilated. Thereafter, the film is lifted and the next filter membrane is promoted to its position. Because of the water repellent quality of the Nuclepore filters, the membrane is almost dry when removed from the support. Subsequently, the film is covered on both sides with an adhesive plastic foil, in order to avoid radioactive contamination. At the exit of the filtration unit, the film is taken up by a film spool.

The described filtration cycle is performed simultaneously with six samples. Figure 5 shows in frontal view the structure of the filtration unit.

The diluter system as well as the pipetting and the filtration unit may also be used at constant temperatures, as checked down to 4 °C. However, most assays, especially the radioimmunoassays for proteo and glycoproteid hormones work perfectly well at room temperature.

[4]) Nuclepore Corporation, Pleasanton, California, U.S.A.

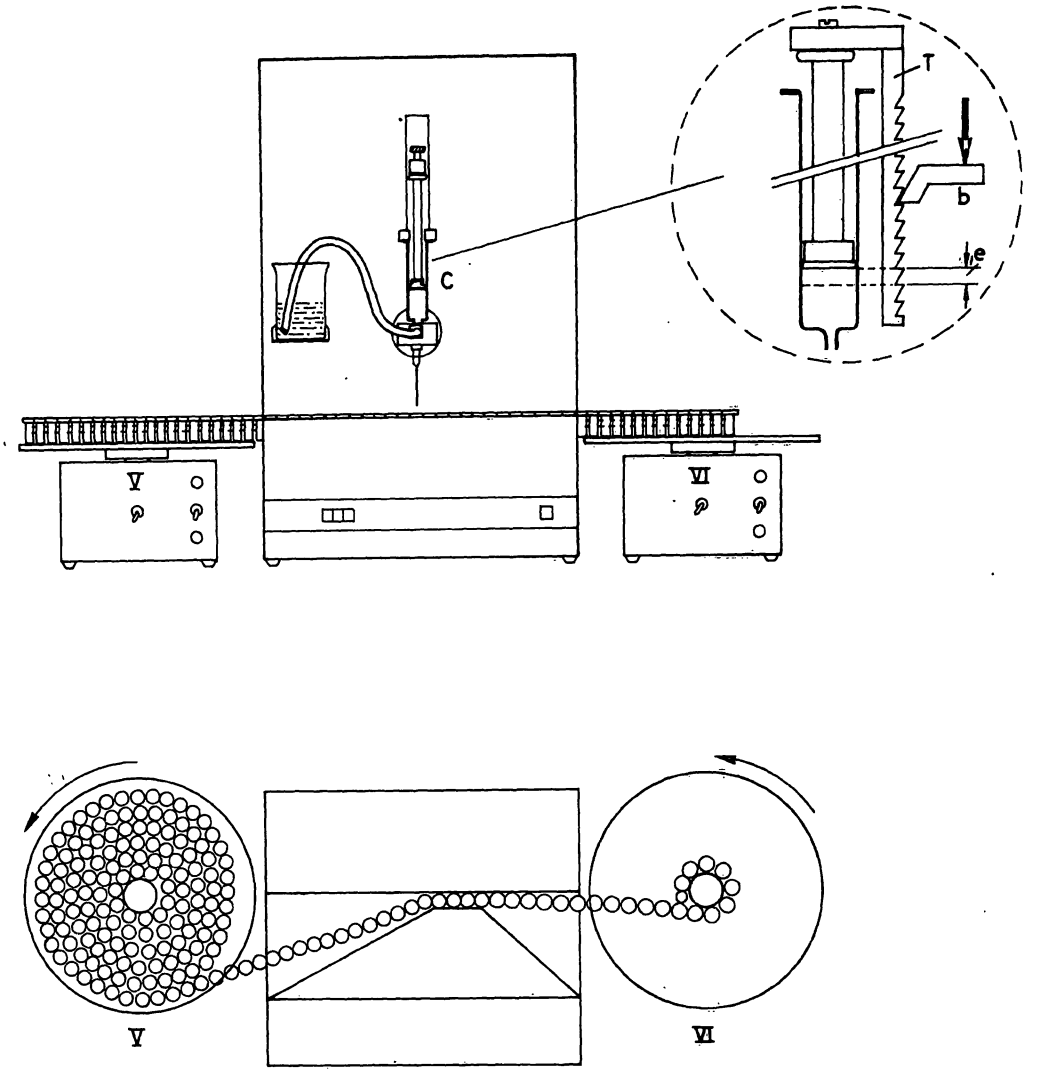

Fig. 3. Pipetting unit, frontal and plan view.

V and VI = induction motors with discs and sample chains. C = Hamilton-gastight syringe (2.5, 5.0 or 10.0 ml). T = toothed rack. b = piston stroke mechanism. e = space of one piston stroke.

Gamma-Counter[5])

The insertion of the film-spool into the gamma-counter replaces the laborious charging of commonly used sample changing counters equipped with well-type crystals. The counter (fig. 6) consists essentially of three pairs of scintillation detectors, each with 3 mm inside width. The crystals cover the radioactive samples almost totally, giving an 8–12% higher counting efficiency than commonly used gamma-counters. The time for the printing of the count rates and for the changing to the next three samples is approximately 5 seconds. A film roll with 800 samples has a diameter of about 14 cm and does not take much space in the radioactive waste deposit.

Calculation of the Standard Curves and the Unknowns

The count rates of the samples are simultaneously printed and punched onto an 8 track paper tape. The latter is subsequently read into a Siemens 404/3 computer with a core of 64 kilobytes. The calculation of the radioimmunoassay standard curve and the evaluation of the unknown hormone concentrations from count rates is performed by smoothing with spline functions (3, 4). The evaluation of an assay with 800 samples, including on-line plotting of the standard curve and printout of the results by a high speed-printer, takes approximately four minutes.

[5]) BF 6000, Labor Professor Berthold, D-7547 Wildbad, FRG.

Results

Results of the evaluation of the accuracy and the precision of the different modules of the analyzer system are listed in table 1. In addition, the table contains information about the carry-over of the pipetting steps. The capacities per hour of the different modules are: 170 to 250 secondary samples for the diluter system depending on the volumes, 1700 samples for the pipetting unit, and 150 to 360 samples for the filtration unit depending on the volumes and the filtration speed, respectively.

The comparison between filtration through cellulose acetate and Nuclepore, respectively, is shown in table 2 for an insulin assay; other radioimmunoassays gave identical results. The Nuclepore material is polycarbonate and has a higher tensile strength and flexibility. Another practical advantage is, that Nuclepore filters must not be premoistened and are water repellent.

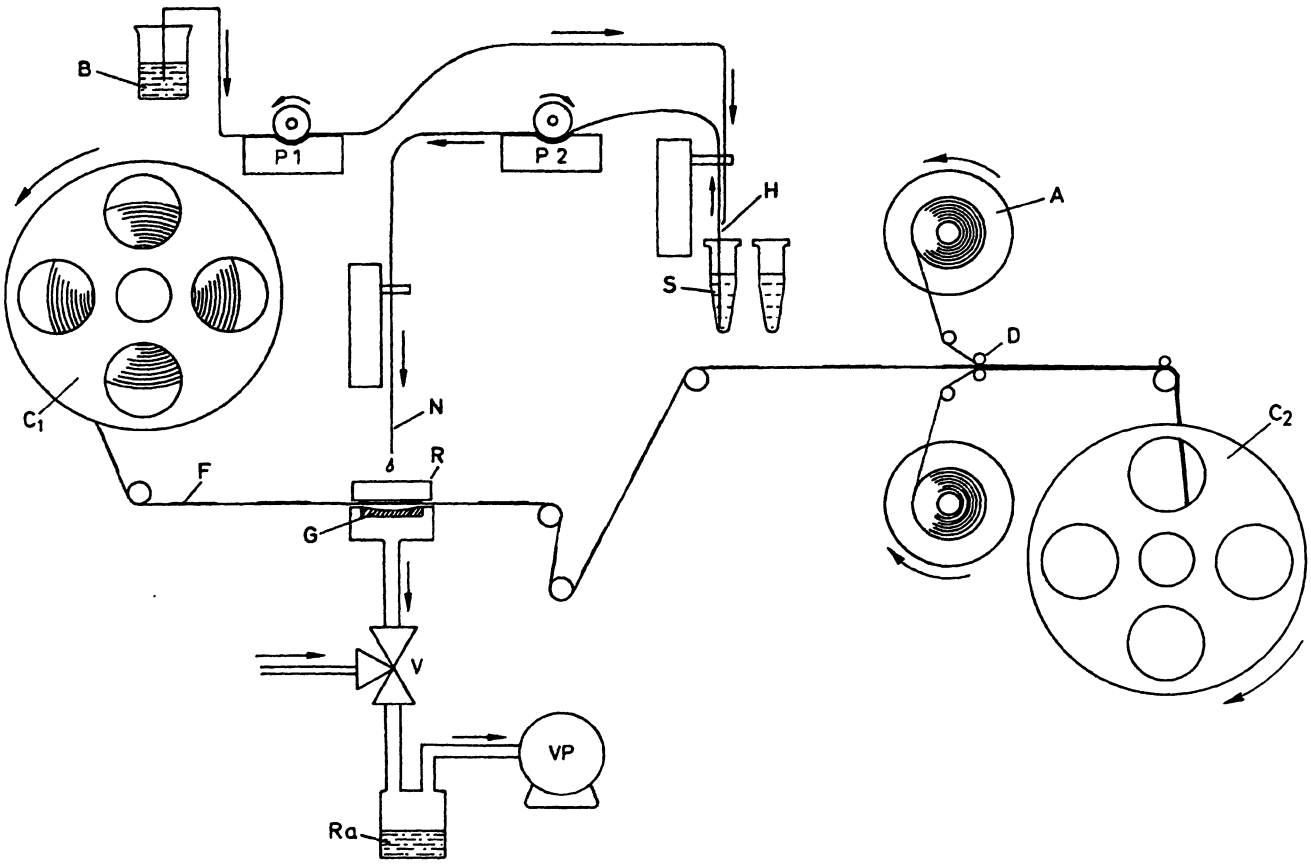

Fig. 4. Schematic representation of one filtration process.

A = adhesive foil. C 1 = spool for stock film. C 2 = spool for covered film, carrying the filters with the bound fractions. D = pressing roll. F = filter. G = concave cut porous glass support for the filter. H = holding device for the sucking and washing needles. N = needle, dropping the sample or washing solution upon the filter.

P 1 = peristaltic pump for B = washing solution. P 2 = peristaltic pump for the sample transport. R = ring, pressing the film around the filter on the glass support during the filtation. Ra = radioactive waste. S = sample in microliter vial. V = magnetic valve. VP = vacuum pump.

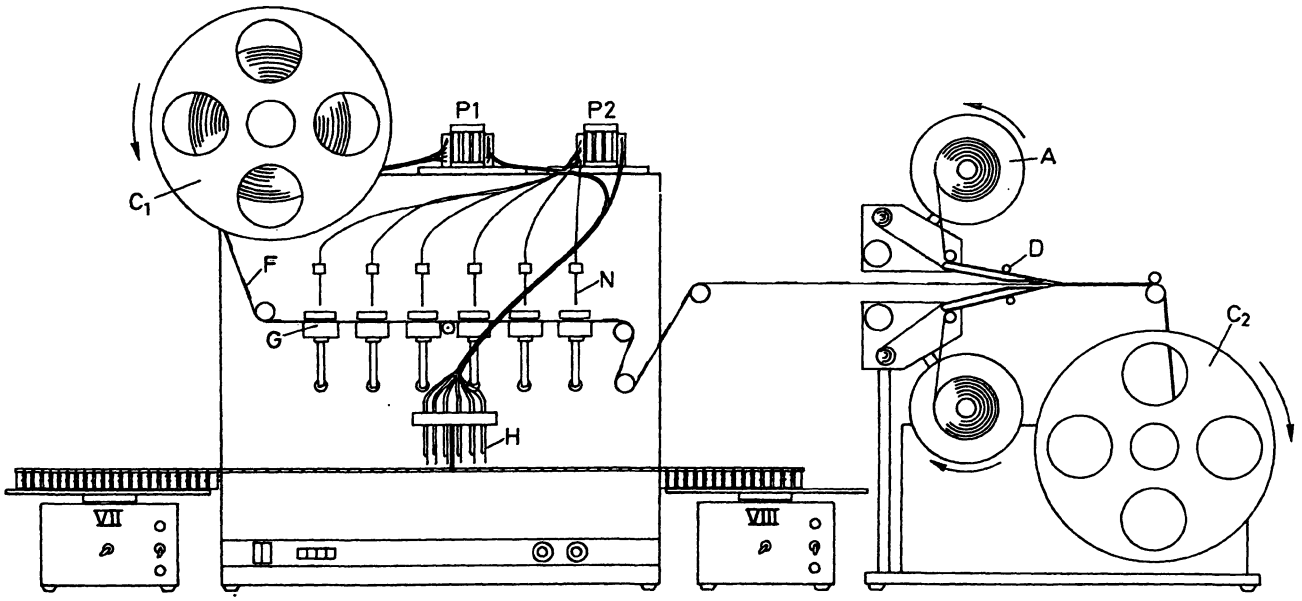

Fig. 5. Frontal view of the filtration unit and the film covering device.

Symbols as in Fig. 4

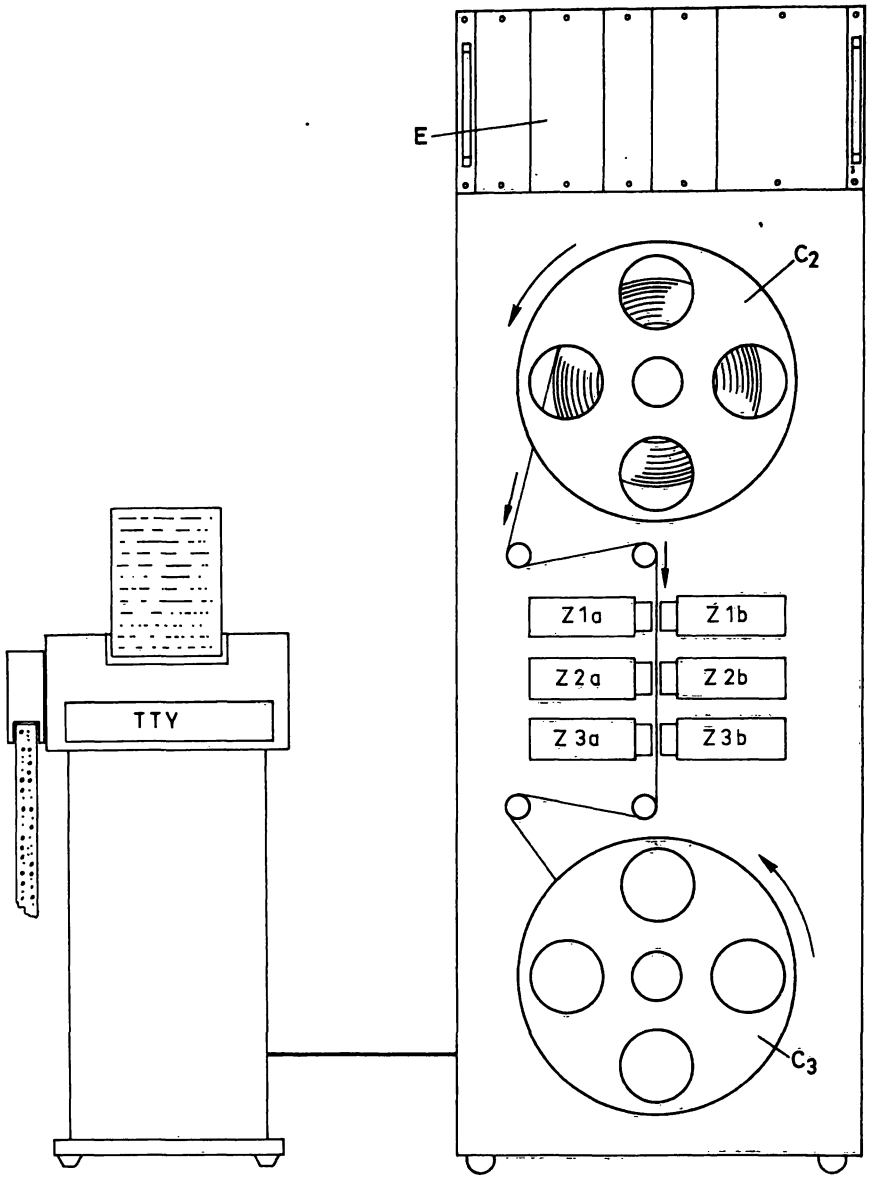

Fig. 6. Gamma-counter for ^{125}I and printer/tape punch.

C 2 = film spool as shown in figure 5, holding the uncounted part of the film. C 3 = film spool receiving the counted part of the film. E = electronic device. Z 1a–Z 3b = three pairs of scintillation crystals. TTY = Teletype with paper tape device.

Tab. 1. 1) Accuracy and precision of the pipetting steps:

adjusted volume	number	mean value (micro balance)	standard deviation	coefficient of variation
a) Diluter system				
300 µl	50	294 µg	1.51 µg	0.53 %
b) Pipetting Unit				
100 µl	150	98 µg	0.86 µg	0.88 %

2) Carry-over in needles and plastic tubings:

a) Diluter System			
sample volume (µl)	100	100	100
buffer volume added (µl)	100	200	300
carry-over (% radioactivity)	2.4	1.0	0.5

b) Filtration unit

The remaining radioactivity in plastic tubes after washing once with 0.6 ml buffer containing 2 g/l bovine serum albumin as measured by using labeled insulin is less than 0.1 %.

The carry-over of radioactivity from one sample with high radioactivity to a subsequent sample without radioactivity using the same filter position and thereby the same tube, is approximately 0.75 %, as measured by the count rates of the corresponding filters.

Tab. 2. Comparison of cellulose acetate[6]) with Nuclepore[4]) filters of different pore sizes.

Assay:	Amersham insulin kit (preprecipitated antibody)				
Cellulose acetate:	10 500 cpm = 100 %				
Nuclepore:	pore size 3.0 µm	1.0 µm	0.6 µm	0.4 µm	0.2 µm
	cpm 855	2136	1664	9657	10059
	% 8	20	16	92	96

Tab. 3. Working instructions for the thyrotropin double antibody radioimmunoassay.

1. Diluter System:

	sample	1st antibody[7])	buffer	number of replicates
zero-standard (B_0)	–	200 µl	100 µl	9
standards[8]) (0.39–100 mU/l)	100 µl	200 µl	–	3
nonspecific binding	–	–	300 µl	6
unknowns	100 µl	200 µl	–	2

Incubation for 60 hours at room temperature.

2. Pipetting Unit:

Addition of 100 µl ^{125}I-labelled thyrotropin[9]) (20000 cpm, specific activity 50 Ci/g) to each vial.

Incubation for 40 hours at room temperature.

3. Pipetting Unit:
Addition of 100 µl 2nd antibody[10]) to each vial.

Incubation for 14 hours at room temperature.

4. Filtration Unit:

Filtration of 6 samples simultaneously (30 s), washing twice with 0.5 ml buffer (50 s), covering of the filters with adhesive foil.

5. Gamma-counter:

Counting of 3 filters simultaneously, printing and punching of sample numbers and count rates.

6. Data Processing:

Punched tape input, calculation and plotting of the standard curve by spline approximation, calculation and printing of sample number, count rate, percent binding, hormone concentration, and limits of confidence for each sample.

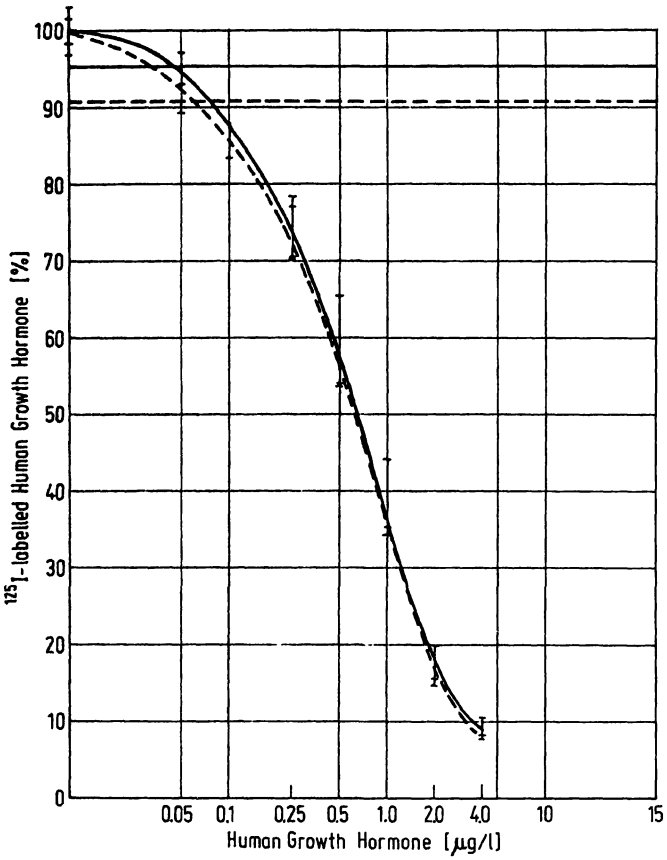

Fig. 7. Correspondence of two human growth hormone standard curves:

solid line = conventional hand pipetting and cellulose acetate filtration, dotted line = analyzer system (compare text). The horizontal lines represent the limits of detection.

So far, the following double antibody assays have been performed with the modular analyzer system described in this paper: thyrotropin, insulin, gastrin, luteinizing hormone, follicle stimulating hormone, growth hormone, and thyroxine binding globulin. Figure 7 shows as an example for all assays the agreement of the two standard curves of an human growth hormone assay, performed with the machine, and performed with conventional hand pipetting and cellulose acetate filtration, using the same reagents. Table 3 contains as an example the working instructions for our routinely performed radioimmunoassay of thyrotropin (5).

Discussion

In most of the larger laboratories which perform radioimmunoassays, the critical length of the series (6) for manual performance is exceeded. This is especially true for those assays, which are used for screening purposes. Generally, mechanization means improvement of the results and reduction in price. Roughly calculated, we assume the critical length with 300 samples (not specimens) per day, which may be derived from different assays. In spite of the increasing numbers of radio-

[6]) Schleicher & Schüll GmbH, D-3354 Dassel, FRG, Nr. CA 250/0
[7]) TSH-antibody (rabbit), op. 137/81171, Behring, Marburg/Lahn, FRG.
[8]) NIMR 68/38, Mill Hill, London, GB.
[9]) TSH for labeling, NIAMD, Bethesda, U.S.A.
[10]) Anti-rabbit precipitating serum (donkey), Deutsche Wellcome GmbH, D-3006 Großburgwedel, FRG.

immunological determinations, only a few systems have been specially developed for radioimmunoassays.

In contrast to the Darias[11]) of *Bagshawe*(7), which also uses the principle of discontinuous filtration, our analyzer system is not on-line controlled by a computer. The on-line operation blocks the expensive computer for other purposes (8). Moreover, the data flow is very slow as compared to the access time of a computer. The term "automated" in Darias is not in agreement with the definition of the "Commission on Nomenclature" of the IUPAC (9), since there is no feed-back mechanism or self-monitoring system controlling the quality of the results and adjusting the machine accordingly. The use of the tray system for the sample transport makes a positive identification (10) in the present stage impossible and does not overcome the "tunnel-effect" (11). The advantage of the use of sample chains is their easy adaptability to the actual number of samples. Both systems are working with the simultaneous counting of more than one sample in the gamma-counting unit. This principle markedly accelerates the speed-limiting step in the whole assay procedure and considerably enlarges the capacity, as compared with commonly used counters.

Currently the problems of positive sample identification are under study. The analyzer system was developed for the use of Eppendorf microliter vials and chains[12]). It is possible, however, by minor modifications, to use coded Silab cups and chains[13]). The following system for positive sample identification is now in development: precoded Silab cups are used for the specimen as well as for the requested number of sample cups. Both are arranged in the same sequence within the primary and secondary chain. Reader devices at the pipetting place of the diluter system control only the identity of the codes. The pipetting unit adds to each sample the same amount of reagent and therefore does not need any decoding. At the filtration unit, the information on each cup is decoded and punched on the film close to the corresponding filter membrane. The information is picked up again in the gamma-counter and is then printed out, and/or punched on the paper tape together with the count rate for the final off-line data processing. There is at no step within the system any disconnection of the samples and their codings so that no so-called tunnel effect appears.

The precision of the modular analyzer system described here corresponds to that of an attentively working technician, however, mix-ups and fatigue do not occur even in large series. The modular construction of the analyzer system permits in itself the independent operation of the four respective units, and allows the full utilization of the capacity and an economical performance of radioimmunoassays.

[11]) "*D*igico *A*utomated *R*adio-*I*mmunoassay *A*nalytical *S*ystem", Digico Ltd., Stemage, Herfordshire, GB.
[12]) Eppendorf Gerätebau, D-2000 Hamburg, FRG.
[13]) Siemens AG., D-8510 Erlangen, FRG.

Acknowledgement

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